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Research Article

Pathogenomic Studies of Streptococcus sanguinis in Infective Endocarditis

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Abstract

***Background**: Infective endocarditis (IE) is a type of endovascular infection caused by bacteria in the bloodstream which get adhered to the damaged heart valve. IE has extensively been associated with oral hygiene, as onset of bacteremia has been observed more often in dental patients. Furthermore, few members of oral microbiota including certain strains of Streptococcus sanguinis have also been isolated from IE colonies. Streptococcus sanguinis is a large group of infectious bacteria including human pathogenic strains. S. sanguinis SK36 is a member of microflora of human mouth and is an opportunistic pathogen of dental plaques. Various other strains of S. sanguinis also constitute human mouth microflora, and have the potential to cause dental plaques, therefore, a possibility is there that they too might have a role in the onset of IE. **Objective:** In this study, we aim to investigate the possible role of all known strains of S. sanguinis in the onset of IE and cluster them accordingly. **Method:** To achieve this, 31 strains of S. sanguinis were investigated and clustered into two groups i.e. Pathogenic and Non-Pathogenic. We further divided the pathogenic cluster into two sub-clusters; moderate and severe pathogens, based on their distance from the reference strain and degree of significance of the virulence determinants they possess. 22 virulence determinants were enlisted by literature survey and revealed association with IE. **Results:** Based on our investigations, we clustered 16 out of 31 strains as pathogenic (4 severe and 12 moderate) and rest of 15 as non-pathogenic of IE. **Conclusion:** It is concluded that not only SK36 and VMC66 but 14 other strains of S. sanguinis also have the potential to cause IE and there is dire need to conduct a comprehensive study to find out their relationship with IE.*

Keywords: Infective Endocarditis, *Streptococcus sanguinis*, IE virulence, Oral diseases, IE pathogens, Oral microbiome

1. Introduction

Infective endocarditis (IE) is a life-threatening endovascular infection caused by adherence of bacteria from the bloodstream to the damaged heart valve (Paik *et al.*, 2005). IE is characterized by the vegetative growth embedded by infection-causing microorganisms along with fibrin and platelets at the site of infection (Cabell *et al.*, 2003, Tilley and Kerrigan, 2013, Bor *et al.*, 2013). This vegetation provides microorganisms with a site to embed and multiply, disrupting normal patterns of blood circulation within the heart, while the intensity and destruction of the tissue depends upon the bacterial species (Shun *et al.*, 2005, McDonald, 2009). In spite of the recent advancements in the medical, surgical and critical care interventions, IE continues to remain a leading cause of morbidity and mortality, not only in West but also in Asian countries like Pakistan where it has the mortality rate of 27.3% (Tariq *et al.*, 2004). Various bacterial pathogenic species including *streptococci*, *staphylococci*, *enterococci* and fastidious *gram negative coccobacilli* have been commonly reported to cause IE (Tilley and Kerrigan, 2013, Tariq *et al.*, 2004). In the recent studies, streptococci specifically *Streptococcus sanguinis SK36* has emerged as the major culprit of IE (Douglas *et al.*, 1993, Paik *et al.*, 2005, Turner, 2008, Ge *et al.*, 2008). *S.sanguinis SK36* is a gram positive bacterium, normally present in the oral microflora in human (Paik *et al.*, 2005, Ge *et al.*, 2008) and is an opportunistic pathogen (Li *et al.*, 2000). From mouth it enters the blood stream through a minor cut or a wound and causes IE. The reason to support this finding is the higher incidence of IE and isolation of *S.sanguinis* in patients

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undergoing dental procedures (Li *et al.*, 2000, Kitten *et al.*, 2012). An association has also been reported between the biofilm forming ability of *S.sanguinis* and the clinical manifestations of IE (Parsek and Singh, 2003).

While the world of literature is rich in research papers spotlighting various aspects of IE, only two *sanguinis* strains have been acknowledged for causing the disease. These strains include SK36 and VMC66, former is the most well-known and well-documented pathogen, while later is also a member of oral microbiota (Paik *et al.*, 2005, Kitten *et al.*, 2012). Various other strains of *sanguinis* such as ATCC29667, SK355, SK330, SK115, SK72, SK353, SK160, SK49, SK340 and SK150 are reported to colonize in human mouth [15]. A very little is known about their pathogenicity because virulence and colonization factors of these strains are still undiscovered.

Thus, there was a need to investigate the pathogenicity of these strains in IE and to affirm the association of dental plaques with the disease. These objectives highlight the need of investigation for examining the pathogenicity of all the available *sanguinis* strains as a pre-requisite and compare them with other members of oral microbiota to validate the role of dental plaques in the IE disease.

This study could serve as the first step towards determining the pathogenicity of *S. sanguinis* strains and could offer better insight into the diagnosis of IE using positive culture technique. It would significantly contribute to the field as *sanguinis* constitutes the largest class of pathogens with unknown pathogenomics (Xu and Ge). Also, determination of previously unknown and unacknowledged pathogens would broader our vision of the spectrum of organisms causing IE and would elucidate their role in the disease.

2. Materials and Methods

We adopted a simple principle of comparison and scanning to achieve the purpose. Adopted approach is shown in Figure 1.

2.1. *Enlisting IE virulence determinants*

Reported infective endocarditis virulence determinants were enlisted with the help of previously published literature (Paik *et al.*, 2005, Tilley and Kerrigan, 2013, Shun *et al.*, 2005, Turner, 2008, Ge *et al.*, 2008, Li *et al.*, 2000, Fan *et al.*, 2012, Rhodes *et al.*, 2014, Viscount *et al.*, 1997, Burnette-Curley *et al.*, 1995, Turner *et al.*, 2009). Information was collected exploiting Polysearch2 and pubmed platforms using multiple keywords such as infective endocarditis, endocarditis, bacteremia, dental plaque, oral microbiome and biofilms.

2.2. *Clustering S. sanguinis strains*

S. sanguinis is a large group of bacteria which includes several human pathogens (Xu and Ge). IE virulent determinants are critical for *S. sanguinis* specie for its pathogenicity. A search of pre-enlisted virulent determinants among other *S. sanguinis* strains could reveal the pathogenic ability of these strains to cause heart infections. Therefore, pathogenomic comparison approach was used to examine the conservation of the virulent determinants within *S. sanguinis* species. 31 *sanguinis* publicly available strains from NCBI genome database were used and were compared through Microbial Protein BLAST (Tatusova *et al.*, 2014) and UniProt (Krislock and Wolkowicz, 2012) to check the presence of the enlisted virulent determinants. Strains were then clustered

into two group i.e. (i) pathogenic strains (ii) non-pathogenic strains, provided that a strain was taken as IE pathogen only if it possessed majority of the enlisted virulent determinants whereas strains short of the virulent determinants were considered non-pathogenic. Pathogenic cluster was further divided into two sub-clusters, "severe" and "moderate" pathogens depending on the following criteria, severe pathogens - least distance a strain had with reference strain *SK36*, more pathogenic it would be. Moderate pathogens – more the distance a strain had with the reference strain; more moderate pathogen it would be. Distance was determined based on the number of virulence determinants a strain possessed with reference to *SK36* and the criteria was developed based on the idea of Euclidean distance i.e.

$d(i,j) = \sqrt{(|x_{i1}-x_{j1}|^2 + |x_{i2}-x_{j2}|^2 + \ldots + |x_{ip}- x_{jp}|^2)}$

(Krislock and Wolkowicz, 2012), where x_i and x_j refers to the total number of virulence determinants possessed by SK36 and strains under investigation respectively. *SK36* was fixed as centroid object for clustering, thus x_i remained fixed, and the number of desired clusters was two. Moreover 1 was taken as a cutoff point for changing the clusters. Role of virulence determinants was not ignored as each virulent determinant do not contribute equally in the onset of the disease. Significance levels of these virulence determinants were found by the examination of previously published literature. Strains included in the study are shown in Figure 4.

2.3. *Pathogenomic comparison*

Comparative genomics approach was exploited to trace the evolutionary relationship between the clustered pathogenic strains. The *SK36* strain was selected as a reference because of its well-known pathogenicity for IE (Nakano *et al.*, 2008) and was compared against the genome of each strain from moderate and severe pathogenic sub-clusters. This genome comparison was achieved using MUAVE and MicroScope tool (Vallenet *et al.*, 2009, Darling *et al.*, 2010).

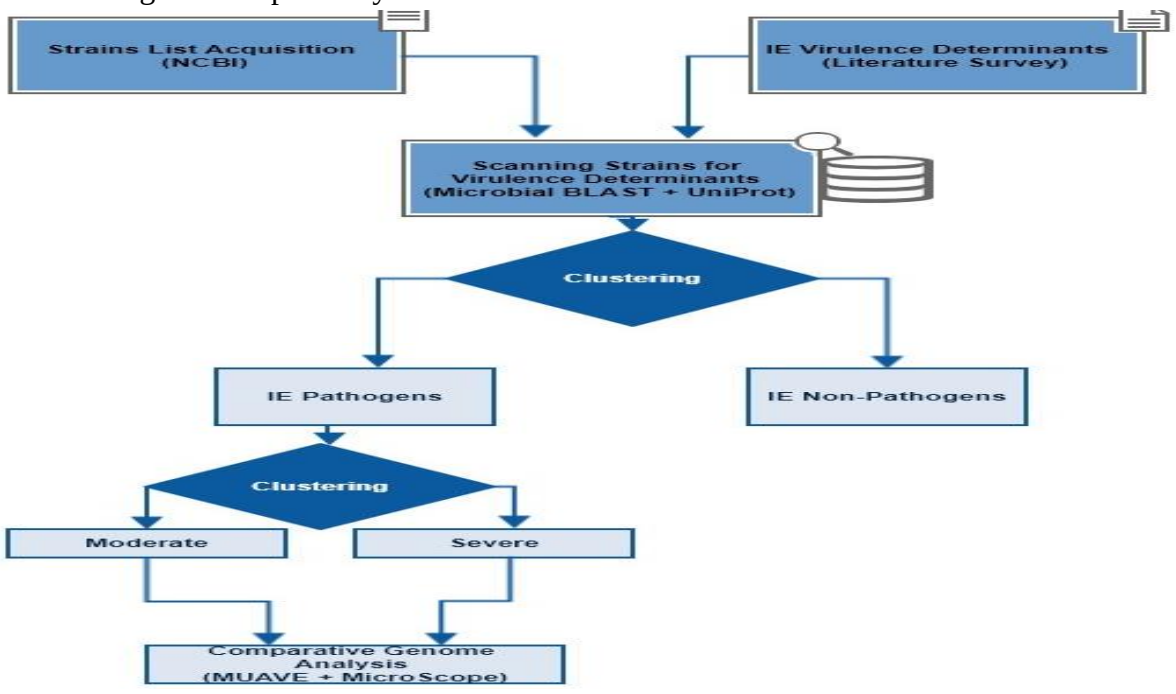


Figure 1: Follow-up methodology for determination of virulent determinants involved in IE

3. Results and Discussion

3.1. *Virulence determinants of IE in S. sanguinis*

Our review of literature revealed 22 reported virulent determinants in *S. sanguinis SK36* associated with IE (Table 1). The prime virulence determinants include cell surface proteins required for the initial stages of the IE (Turner, 2008). Cell wall anchoring proteins were found to be important in pathogenesis of IE. Many have been grouped as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) of host tissues and cells (Turner, 2008). Among MSCRAMMs, clumping factor fibronectin binding proteins A (FbpA) was an important pathogenic factor (Turner, 2008). FbpA was found to be capable of binding to fibronectin which exist in the extracellular matrix of most tissues in hosts. Streptococcal MSCRAMMs identified as mediators of IE include CpbA of *S. sanguinis*. CbpA is collagen binding surface protein and is recognized as a moderator of platelet aggregation in vitro (Paik *et al.*, 2005, Turner, 2008).

In addition to that, different rhamnose-glucose polysaccharide RGPs also contribute to the virulence of IE because of their adhesive properties to collagen type I and laminin. RGPs can bind and activate platelet aggregation which leads to further complications in IE. RGPs observed in SK36 were rgpB, rgpD, rgpG, rgpF, rgpA, rgpC and rgpFc. Such proteins play an important role in initial bacterial binding to the blood vessels (Turner, 2008, Nakano *et al.*, 2008).

Extracellular production of glucan polymers was also linked to IE infectiom. The extracellular glucans are synthesized by bacterially encoded glucosyltransferase (Gtf) enzymes from a sucrose substrate and enhance colonization in the development of IE. Studies suggest that glucan production enhance streptococcal survival post-phagocytosis putatively mediating adherence to vegetation-like matrices and includes gtfA, gtfB and gtfP from SK36 (Paik *et al.*, 2005, Shun *et al.*, 2005, Nakano *et al.*, 2008, Hinse *et al.*, 2011). FimA, another important virulent gene is associated with initial colonization of damaged heart tissue, and is associated with manganese and iron uptake in IE model (Paik *et al.*, 2005, Hinse *et al.*, 2011, Burnette-Curley *et al.*, 1995).

Studies have also reported a link between ability of *S. sanguinis* biofilm formation and the clinical manifestations of IE (Parsek and Singh, 2003). This implies that biofilm forming genes such as purB, bacA, thrB and thrC found in *S.sanguinis* play their virulent role in IE (Paik *et al.*, 2005). Mutation in purB attenuates the biofilm formation as well as the IE whereas purB, purL and pyrE are required for bacterial survival in blood and hence play indirect role in the onset of IE (Ge *et al.*, 2008). The genes thrB and thrC encode homoserine kinase i.e. enzyme that catalyzes the first committed step for synthesis of threonine and isoleucine.

Nt5e is also dominant virulence factor that plays important role in inhibiting phagocytic monocytes/macrophages associated with valvular vegetation and promotes the survival of *S. sanguinis*. It also functions in the inhibition of platelet aggregation that could delay presentation of platelet microbicidal proteins to heart valve infecting bacteria (Fan *et al.*, 2012).

Ribonucleotide reductases (RNRs) that convert ribonucleotides to the deoxyribonucleotides involved in DNA replication and repair mechanisms are

essential for the *sanguinis* to survive anaerobic conditions. Among RNP encoding genes, nrdE and nrdF have been marked essential for causing IE as mutation in any of these genes completely abolish the IE virulence ability of *sanguinis* strains (Rhodes *et al.*, 2014).

Table 1: IE virulence genes catalog along with functions, locus tags and sub-cellular locations

S.No	Locus tag	Gene Name	Start	Stop	Function	SCL
1	SSA_0907	fpbA	917658	919307	fibronectin binding protein	Cytoplasmic Membrane
2	SSA_1663	cpbA	1659676	1664211	collagen binding surface protein	Cell wall
3	SSA_1234	Nt5e	1257726	1259885	inhibits phagocytosis	Cell wall
4	SSA_1508	rgpC	1512534	1513340	activate platelet aggregation	Cytoplasmic Membrane
5	SSA_1506	rgpFc	1509642	1511312	activate platelet aggregation	Cytoplasmic
6	SSA_1510	rgpA	1514278	1515426	activate platelet aggregation	Cytoplasmic
7	SSA_1513	rgpF	1518085	1521066	activate platelet aggregation	Cytoplasmic Membrane
8	SSA_1507	rgpD	1511329	1512534	activate platelet aggregation	Cytoplasmic Membrane
9	SSA_1957	rgpG	1852815	1853177	activate platelet aggregation	Cytoplasmic Membrane
10	SSA_1509	rgpB	1513346	1514281	activate platelet aggregation	Cytoplasmic Membrane
11	SSA_0613	gtfP	601987	606693	post-phagocytosis streptococcal survival	Extracellular
12	SSA_1006	gtfA	1007216	1008661	post-phagocytosis streptococcal survival	Cytoplasmic
13	SSA_0838	gtfB	825546	826889	post-phagocytosis streptococcal survival	Cytoplasmic Membrane
14	SSA_1240	pyrE	1264891	1265520	promotes bacterial survival	Cytoplasmic
15	SSA_0046	purB	51034	52329	biofilm formation	Cytoplasmic Membrane
16	SSA_0030	purL	32750	36484	promotes bacterial survival	Cytoplasmic
17	SSA_1959	bacA	1955679	1956521	biofilm formation	Cytoplasmic Membrane
18	SSA_0095	thrC	96695	98179	biofilm formation	Cytoplasmic
19	SSA_1044	thrB	1059993	1060859	biofilm formation	Unknown
20	SSA_0770	nrdE	749342	751501	promotes survival in anaerobic conditions	Cytoplasmic
21	SSA_0768	nrdF	748226	749185	promotes survival in anaerobic conditions	Unknown
22	SSA_1633	FimA	1634196	1635635	initial colonization of damaged heart tissue	Cellwall

3.2 *Clustering of S. sanguinis strains*

Pre-enlisted virulent determinants were scanned in the genome of the strains for the first round of clustering. Results indicated the presence of virulent determinants in 22 out of 31 *sanguinis* strains and complete absence in rest of the nine strains (Figure 2). Strains that possess virulent genes include

SK36, VMC66, SK353, SK405, SK678, SK2, SK115, SK150, SK160, SK1057, SK330, SK408, SK1058, SK1087, SK1059, SK49, SK1056, SK355, SK340, ATCC29667, CC94A and NCTC7863. However, virulence determinants were found missing in 216SSAN, 711SSAN, MB451, FSS4, FSS9, PJM8, 2908, 1112SS and SK1. Based on these results, 22 strains were clustered as pathogenic whereas 9 were grouped as non-pathogenic. Genomes of strains constituting pathogenic cluster was subjected to further investigation, depending upon the contribution of each virulent gene in the onset of IE. Along with that "contribution model of virulent genes towards the dentally associated IE" has also been proposed (Figure 3). This model is based on previously proposed "causal model of dentally associated endocarditis" from Li et.al and function of each gene as discussed in section 3.1.

Pathogenic cluster was further divided into the sub-clusters of severe and moderate pathogens based on the distance of these strains from the centroid strain SK36. Four strains of the pathogenic cluster showed zero distance with the centroid and were put into the cluster named as severe pathogens. These strains include SK36, VMC66, SK353 and ATCC2966. These strains possess all of the virulent genes of SK36 which suggest them to be hyper-virulent. Whereas, distance of the rest of the 9 pathogenic strains in reference to the centroid was > 0 which indicates absence of atleast one virulence determinant that makes these strains moderate pathogens.

Results revealed absence of FimA in most of the strains. FimA is involved in the initial binding to the blood vessels was missing in SK405, SK678, SK150, SK160, SK330, SK1057, SK408, SK115, SK1058, SK49, SK1056, and CC94A strains. Thus these strains possess reduced ability to cause IE as studies have indicated strains with mutant FimA reduces the ability of *S. sanguinis* to cause IE (Hinse *et al.*, 2011, Viscount *et al.*, 1997, Burnette-Curley *et al.*, 1995).

On the second highest frequency, strains lacked CpbA that is mediator of platelet aggregation. These strains include SK72, SK115, SK1058, SK1059, SK1087, SK1056, SK355, SK340 and CC94A.These strains do not completely lose the ability of causing IE as studies have shown strains continues to be infectious even when any of cwa is mutated (Turner, 2008, Turner *et al.*, 2009).

On the third highest frequency, gtfA was found absent in the strains. These strains include SK405, SK1057, SK408, SK1058, SK1087, SK49 and CC94A. Similarly, gtfB was absent in SK1059 and rpgG in SK340. Absence of gtfA, gtfB and rpgG also decreases the ability of strains to cause IE.

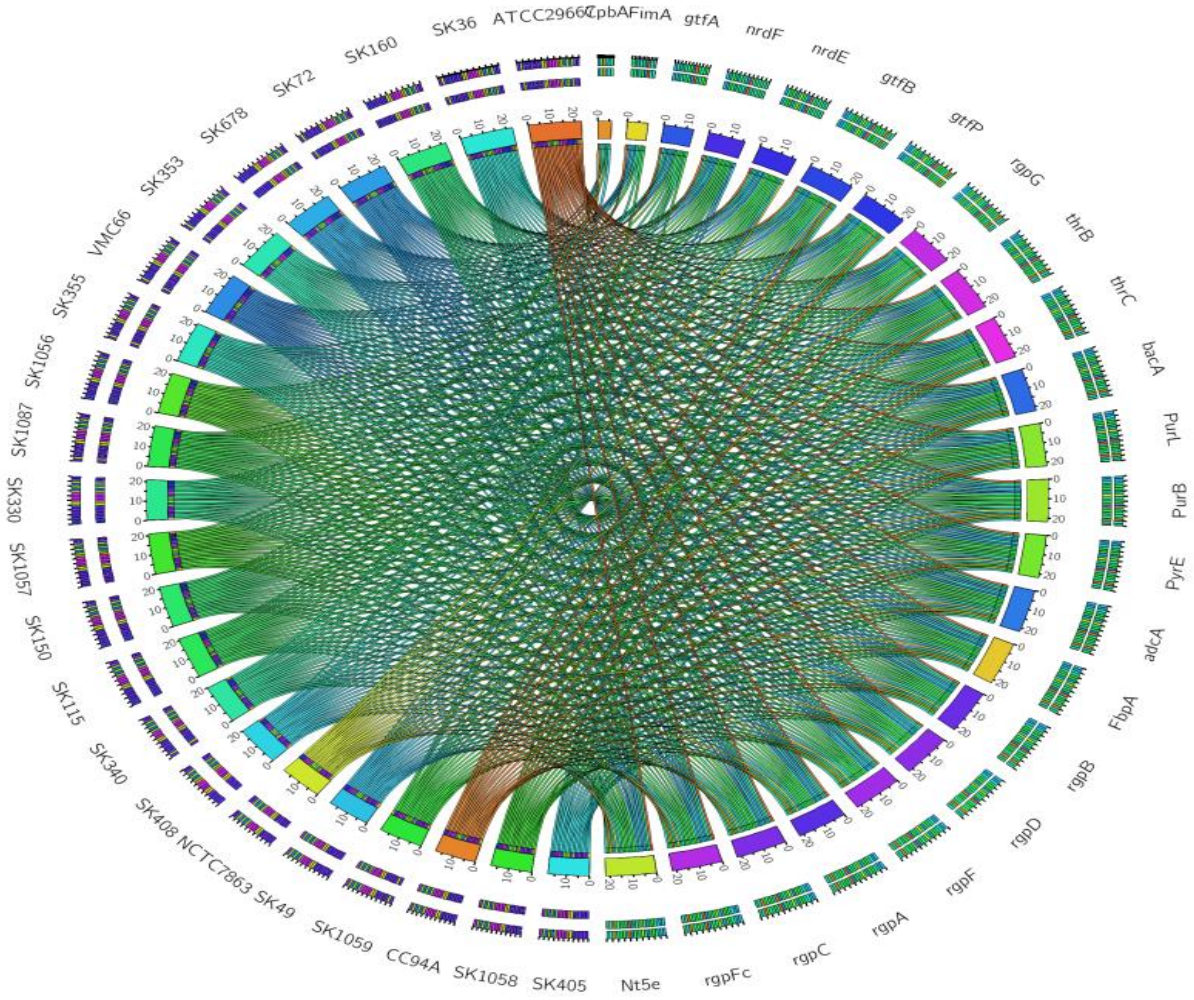
Fourth highest frequency was of nrdE which was found absent in SK405, SK1058, SK1059, SK49 and CC94A. Then was nrdF which was found absent in SK405, SK1058, SK1059, CC94A and NCTC7863. Absence of nrdE and nrdF (collectively referred as nrdEF) minimizes the ability of these strains to cause IE (Rhodes *et al.*, 2014). Thus, absence of nrdEF made SK1058, SK1059, CC94A and SK405 non-pathogenic in context to IE where as SK49 and NCTC7863 remain pathogenic because of the presence of any one of the nrdEF.

Because of two rounds of clustering, 6 out of 22 pathogenic strains were excluded from the pathogenic cluster in the process of sub-clustering and the number of non-pathogenic strains became 9+6=15. Among rest of the

16 pathogenic strains, 4 strains were grouped as "severe pathogens" and 12 as "moderate pathogens" of IE. Clustering is shown in Figure 4.

Interestingly, we found that the sub-cluster "severe pathogens" include strains that constitute oral microbiome. These strains include SK36, VMC66, SK353 and ATCC29667. Whereas, sub-cluster of "moderate pathogens" include combination of both and include 6 members of oral microbiome and 6 other strains. Members of oral microbiota included in this sub-cluster are SK330, SK340, SK355, SK115, SK150 and SK72. These findings affirm the role of dental plaques in some cases of IE whereas; on the other hand, it also brings to light certain strains that could potentially cause IE without being associated with dental procedures. However, further advance studies are required to get insight into the reason behind this changing trend.

Figure 2: Presence of virulent gene in *S. sanguinis* strains



This figure represents presence of virulent genes in all the strains. Circularly arranged segments, whose length is proportional to the total cell values in a row or column, represent genes and strain names. Central is the encoding of cell values using ribbons that connect the row and column segments. Whereas the three outer rings are stacked bar plots that represent relative contribution of a cell to row and column totals. Figure was drawn using circos available at (http://www.circos.ca/) on 31st March, 2018.

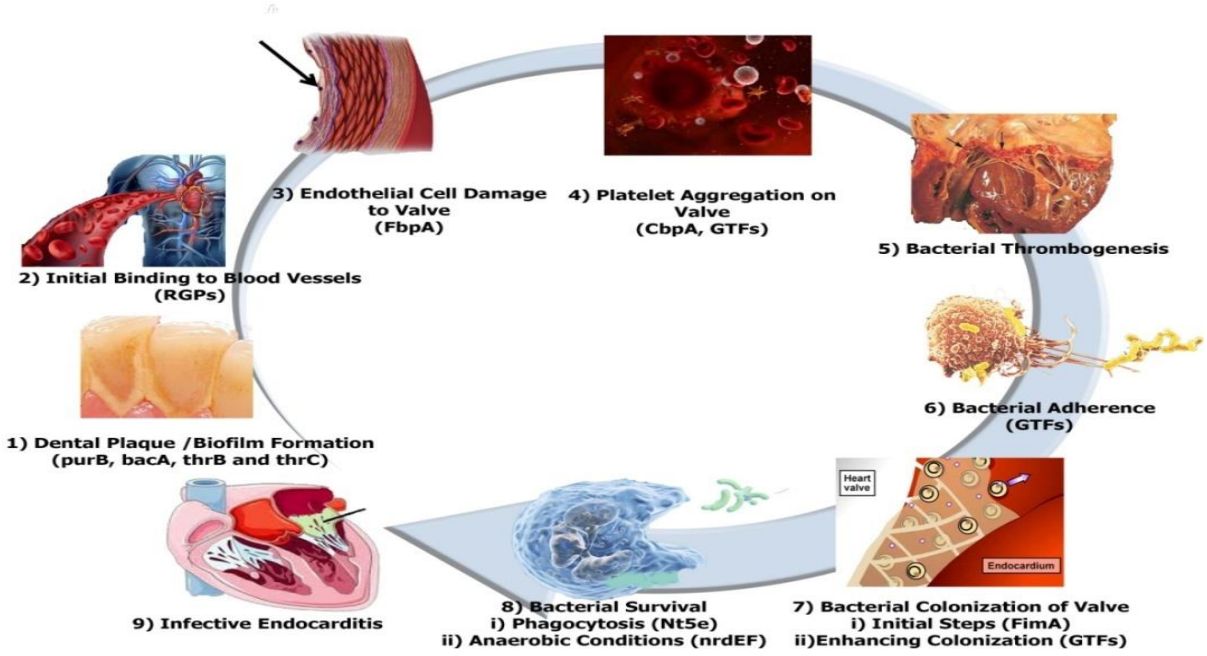


Figure 3: Contribution of virulent determinants towards the dentally associated IE

This figure represents role of discussed IE virulent determinants in each step leading to IE.

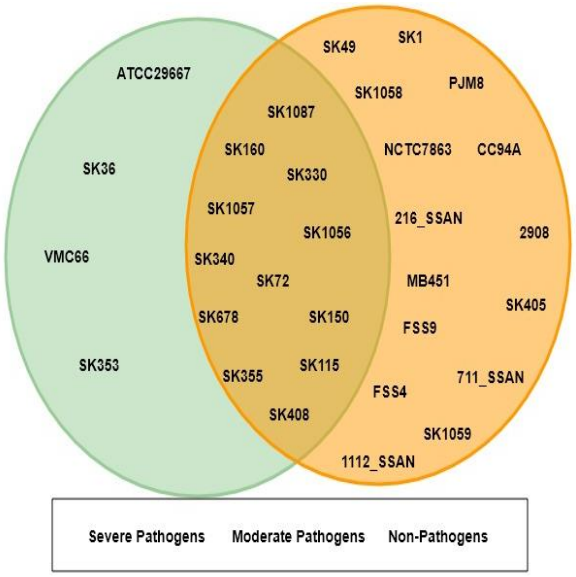


Figure 4: Classification of *S. sanguinis* strains based on their pathogenic association with IE

Pathogenic group includes strains that possess IE virulent genes whereas non-pathogenic strains do not. Overlapped area contains strains lying between severe pathogens and non-pathogens in their pathogenicity, hence, moderate IE pathogens.

3.3. *Comparative genome analysis of pathogenic strains*

In a direct comparison of "IE severe pathogens" (VMC66, SK353 and ATCC29667) with reference to SK36, 75% to 80% of the CDS are found conserved. Whereas 6% to 16% are strain specific CDS. Genome structure and arrangement is not much similar as certain genome re-arrangement of significantly larger genome segments is observed (Figure 6). The comparison of VMC66 with SK36 yielded 1817 core, 464 variable and 151 strain specific CDS. SK353 showed 1816 core, 453 variable and 137 strain specific CDS when compared with SK36 whereas ATCC29667 gave 1822 core, 588 variable and 398 strain specific CDS when compared with SK36 (Table 2). Results therefore suggest more genome conservation in this sub-group of pathogenic strains and numerous strain specific regions which suggest fewer functional genes have originated by genetic evolution or lateral gene transfer (LGT) in these strains.

Table 2: Comparative genome results of severe IE pathogens

Strains	Total CDS	Core	Variable	Strain Specific
SK36	2379	1821	558	239
VMC66	2284	1817	464	151
ATCC29967	2410	1822	588	398
SK353	2272	1816	453	137

Comparison of genomes of "severe IE pathogens" and alignment was performed to get insight into the genomic regions of similarity. High similarity was revealed between all four strains of severe pathogens sub-cluster but with certain genomic rearrangement events. These inversion events were observed in all three strains (VMC66, SK353 and ATCC29667) relative to SK36. In VMC66, we observed genomic co-ordinates from 700000 to 2300000 to be in reverse complement (inverse) orientation, which suggests large inversion event in this genome. In SK353, an inversion event was observed in the genome sequences from 100000 to 1800000. In ATCC29967, three inversion events were observed 100000 to 1400000, 1800000 to 1870000, and 2330000 to 2400000.

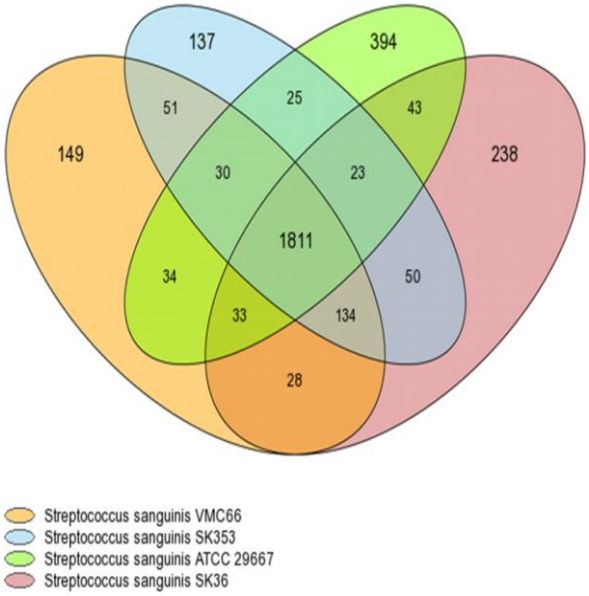


Figure 5: Comparative genome analysis of severe IE pathogens

Venn diagram represents the genetic sharing between severely pathogenic strains of IE.

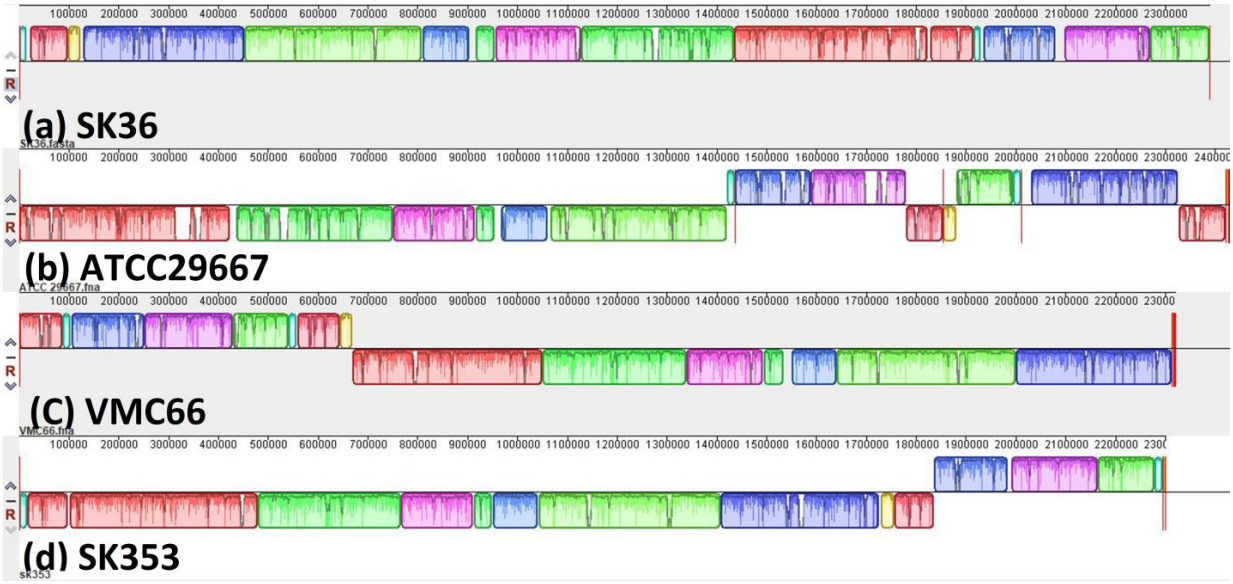


Figure 6: Multiple alignment of severe IE pathogens

Above figure shows an alignment of VMC66, SK353 and ATCC29667 with reference to SK36. Each of these colored block's outlines surrounds a region of the genome sequence that is aligned to part of another genome, and is presumably homologous whereas white regions in the block specifies strain specific regions. Blocks above the center line represents aligned regions is in the forward orientation

relative to the first genome whereas blocks below the center line indicate regions that align in the inverse orientation. Regions outside blocks lack detectable homology among the input genomes. Figure was drawn using MUAVE on 8th April, 2018.

In a direct comparison of "IE moderate pathogens", ≥74% of the CDS were observed to be conserved between these strains which is a significant percentage whereas ≥25% are variable CDS. Diversity in arrangement of genetic information was evident in these strains and certain genome re-arrangement events randomly distributed along the genomes were observed (Figure 7). Genome comparison results of "moderate pathogens" with SK36 are shown in detail in Table 3. Results indicated that more variable CDS are found in this sub-group and significantly higher number of strain specific CDS, which differentiate these pathogens from SK36 and validate their grouping as moderate pathogens.

Table 3: Comparative genome results of moderate IE pathogens

Strain	CDS	Core	Variable	Strain Specific
SK678	2247	1670	575	63
SK115	2290	1669	619	117
SK160	2300	1670	626	81
SK150	2311	1670	639	151
SK1087	2319	1672	644	117
SK355	2326	1671	652	161
SK1057	2337	1669	664	94
SK72	2355	1671	682	84
SK340	2366	1670	694	152
SK1056	2376	1671	703	240
SK330	2385	1670	712	129
SK408	2392	1671	718	160

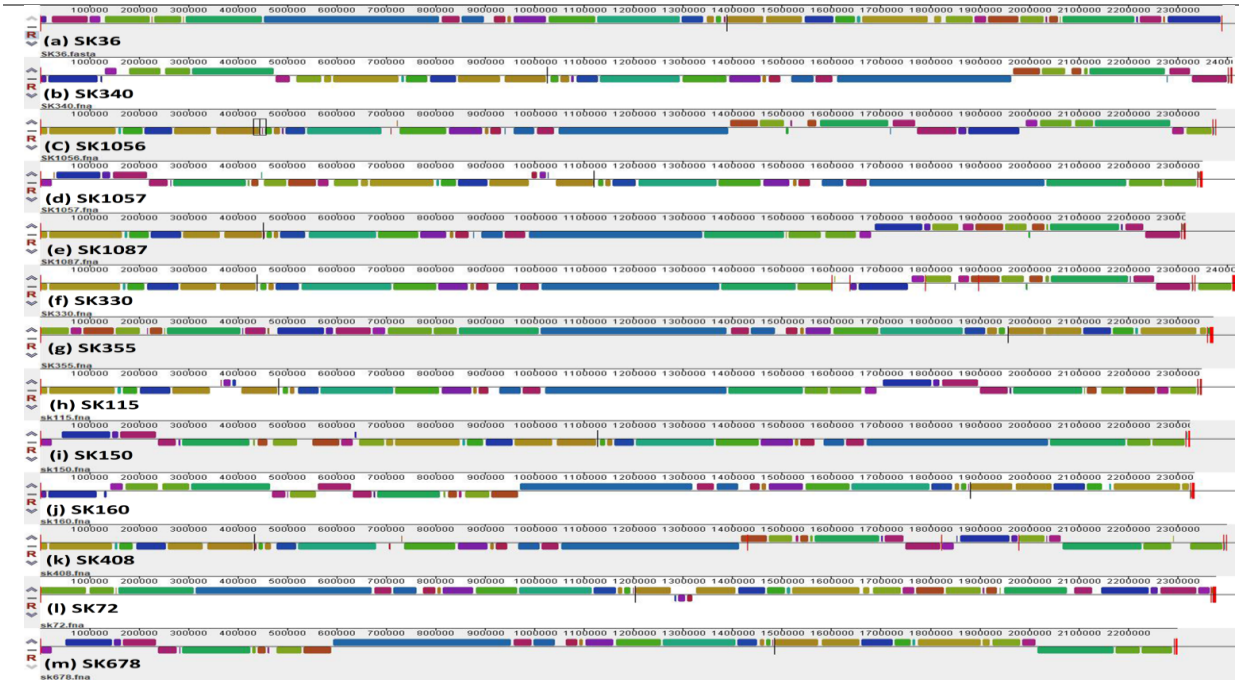


Figure 7: Multiple alignment of moderate IE pathogens

Above figure shows an alignment of 12 strains (moderate pathogens) with reference to SK36. Each of these colored block's outlines surrounds a region of the genome

sequence that is aligned to part of another genome, and is presumably homologous. Blocks above the center line represents aligned regions is in the forward orientation relative to the first genome whereas blocks below the center line indicate regions that align in the inverse orientation. Regions outside blocks lack detectable homology among the input genomes. Figure was drawn using MUAVE on 10th April, 2018.

4. Conclusion

It is concluded that not only SK36 and VMC66 but 14 other strains of *S. sanguinis* also have the potential to cause varying degree of IE that has broaden up the spectrum of organisms causing bacterial endocarditis. Furthermore, not only members of oral microbiome cause IE and that role of *sanguinis* strains other than those that are dentally associated is also important in the onset of the disease. The genomes of severe pathogens show 80% similarity with SK36 whereas moderate pathogens show lower. Numbers of inversion events have also been observed in the strains of both sub-clusters that require further studies to trace the evolutionary basis of the pathogenicity. This study has provided biologists with the first step towards determining the pathogenicity of *S. sanguinis* strains that is still undiscovered. Determining the pathogenicity of these strains could lead to finding out the missing links with certain important infectious diseases that are needed to be acknowledged.

5. Conflict of Interest

Authors declared no conflict of interest.

6. Acknowledgement

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